EFFECTS OF TUALANG HONEY ON KAINIC ACID-INDUCED MORPHOLOGICAL CHANGES AND GLUTAMATE TRANSPORTER (EAAT2) EXPRESSION IN THE CEREBELLUM AND STRIATUM OF RATS

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by

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Stay safe and together we fight COVID-19! Thank you.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
α	alpha
µ	micro
^{o}C	degree Celcius
AED	anti-epileptic drug
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
ARASC	Animal Research and Service Centre
ATP	adenosine triphosphate
BSA	bovine serum albumin
Ca^{2+}	calcium ion
cm	centimetre
CNS	central nervous system
D	days
DAB	3,3'-diaminobenzidine
dH_2O	distilled water
DPX	dibutylphthalate polysterene xylene
EAAC 1	excitatory amino acid carrier 1
EAAT	excitatory amino acid transporter
EDTA	ethylenedinitrilotetraacetic acid
ER	endoplasmic reticulum
FAMA	Federal Agricultural Marketing Agency
g	gram
GABA	gamma-aminobutyric acid
GLAST	glutamate aspartate transporter
GLT-1	glutamate transporter 1
GS	glutamine synthetase
H	hour
H&E	haematoxylin and eosin
HCl	hydrochloric acid

HRP	horseradish peroxidase
i.p.	intraperitoneal
IHC	immunohistochemistry
KA	kainic acid
KAR	kainate receptors
kg	kilogram
L	litre
m	metre
mg	milligram
mL	millilitre
mm	millimetre
Na^+	sodium ion
Na_2HPO_4	disodium phosphate
NaCl	sodium chloride
NaH_2PO_4	Monosodium phosphate
NaOH	sodium hydroxide
NDD	neurodegenerative diseases
NMDA	N-methyl-D-aspartic-acid
NMDAR	N-methyl-D-aspartic-acid receptor
OFT	open field test
PBS	phosphate buffer saline
PFA	paraformaldehyde
ROS	reactive oxygen species
s.c.	subcutaneous
TBS	Tris-buffered saline
TH	Tualang honey
TPM	topiramate
Tris	tris(hydroxymethyl)aminomethane
USA	United State of America
USM	Universiti Sains Malaysia
v/v	volume per volume
w/v	weight per volume

LIST OF APPENDICES

KESAN MADU TUALANG TERHADAP PERUBAHAN MORFOLOGI DAN EKSPRESI PENGANGKUT GLUTAMAT (EAAT2) ARUHAN ASID KAINIK PADA SEREBELUM DAN STRIATUM TIKUS

ABSTRAK

Eksitotoksisiti adalah sejenis kematian sel neuron yang disebabkan oleh glutamat atau asid amino rangsangan yang berlebihan dan merupakan punca kepada pelbagai penyakit neurodegeneratif. Pengangkut asid amino rangsangan 2 (EAAT2) adalah pengangkut glutamat utama yang bertanggungjawab untuk hampir 90% pengambilan semula glutamat di dalam otak. Kekurangan EAAT2 menyebabkan pengumpulan glutamat ekstraselular dan seterusnya eksitotoksisiti. Madu lebah Tualang (TH) adalah madu Malaysia yang telah menunjukkan banyak kesan yang bermanfaat dalam pelbagai model penyakit. Objektif utama kajian ini adalah untuk menilai potensi kesan perlindungan madu Tualang terhadap perubahan morfologi dan ekspresi EAAT2 akibat aruhan asid kainik dalam cerebellum dan striatum tikus. Sejumlah 48 ekor tikus jantan Sprague-Dawley dewasa dengan berat 260 - 320g dibahagikan secara rawak kepada empat kumpulan utama (n = 12 setiap kumpulan) bergantung kepada rawatan yang diterima: kawalan, KA, TH+KA dan TPM+KA. Setiap kumpulan utama kemudian dibahagikan kepada dua subkumpulan bergantung kepada masa korban (24 jam atau 5 hari selepas pemberian KA) (n = 6 setiap subkumpulan). Tikus-tikus tersebut dirawat secara oral dengan air suling (kumpulan kawalan dan KA), madu Tualang (1.0 g/kg; kumpulan TH+KA) atau topiramat (40 mg/kg; kumpulan TPM+KA) selama lima kali pada selang 12 jam. Tikus kemudiannya disuntik secara subkutin (bawah kulit) dengan KA (15 mg/kg; kumpulan KA, TH+KA and TPM+KA) atau larutan garam (kumpulan kawalan) 30 minit selepas rawatan oral terakhir. Ujian lapangan terbuka (*open field test*) dilakukan untuk menilai aktiviti lokomotor tikus sebelum tikus dikorbankan pada 24 jam atau 5 hari selepas pemberian

KA. Serebellum dan striatum dikumpulkan untuk penilaian histologi dan EAAT2. Pengurangan ketara bilangan neuron dan ekspresi EAAT2 telah diperhatikan dalam serebelum dan striatum 24 jam berikutan pemberian KA. Peningkatan ketara dalam aktiviti lokomotor, bersama dengan pengurangan ketara bilangan neuron di dalam serebelum dan ekspresi EAAT2 di dalam striatum telah diperhatikan 5 hari selepas pemberian KA. Pra-rawatan dengan TH meningkatkan bilangan neuron hidup dan EAAT2 di dalam serebelum dan striatum. Kesannya adalah setanding dengan TPM, ubat kawalan yang digunakan dalam kajian ini. Penemuan ini mencadangkan bahawa pra-rawatan dengan TH menunjukkan kesan perlindungan terhadap eksitotoksisiti yang disebabkan oleh KA melalui modulasi ekspresi EAAT2.

EFFECTS OF TUALANG HONEY ON KAINIC ACID-INDUCED MORPHOLOGICAL CHANGES AND GLUTAMATE TRANSPORTER (EAAT2) EXPRESSION IN THE CEREBELLUM AND STRIATUM OF RATS

ABSTRACT

Excitotoxicity is a type of neuronal cell death induced by excessive glutamate or other excitatory amino acids and has been implicated in various neurodegenerative diseases. The excitatory amino acid transporter 2 (EAAT2) is a major glutamate transporter responsible for nearly 90% of glutamate reuptake in the brain. Loss of EAAT2 causes accumulation of extracellular glutamate and excitotoxicity. Tualang honey (TH) is a Malaysian honey that has shown many beneficial effects in various disease models. The main objectives of this study were to evaluate the potential protective effects of Tualang honey on kainic acid-induced morphological changes and EAAT2 expression in the cerebellum and striatum of rats. A total of 48 male adult Sprague Dawley rats with the weight of 260-320g were randomly divided into four major groups (n = 12 per group) depending on the treatment received: control, KA, TH+KA and TPM+KA. Each major group was further divided into two subgroups depending on sacrifice time (24 hours or 5 days following KA administration) (n = 6 per subgroup). The rats were pre-treated orally with distilled water (groups control and KA), Tualang honey (1.0 g/kg; group TH+KA) or topiramate (40 mg/kg; group TPM+KA) for five times at 12 hours interval. The rats were then injected subcutaneously with KA (15 mg/kg; groups KA, TH+KA and TPM+KA) or normal saline (control) 30 minutes after the last oral treatment. An open field test was performed to assess the locomotor activity of rats before the rats were sacrificed at 24 hours or 5 days after the KA administration. The cerebellum and striatum were collected for histological and EAAT2 assessment. Significant reduction in the number

of viable neurons and EAAT2 expression were observed in both cerebellum and striatum 24 hours following KA administration. KA-induced significant increase in locomotor activity, along with significantly reduced viable neurons in the cerebellum and EAAT2 expression in the striatum were observed 5 days following KA administration. Pre-treatment with TH increased the number of viable neurons and EAAT2 expression in the cerebellum and striatum. The effects were comparable to TPM, the control drug used in this study. These findings suggest that pre-treatment with TH showed some protective effects against KA-induced excitotoxicity via modulation of EAAT2 expression.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Neurodegenerative diseases are a group of neurological disorders associated with irreversible loss of neuronal structure and subsequent functional impairment, which eventually results in neuronal cell death (Akbar et al., 2016; Fan et al., 2017). Neurodegenerative diseases can be classified based on their primary clinical presentations, anatomical region of neurodegeneration or abnormality in cellular and molecular levels (Dugger and Dickson, 2017). Common clinical manifestations include movement disorders, cognitive dysfunction as well as behavioral disorders. Intracellular and extracellular accumulations of misfolded proteins and their anatomical distribution are essential for their clinical presentations and diagnosis (Dugger and Dickson, 2017; Kovacs et al., 2017).

For example, the cerebellum and striatum are major subcortical nuclei brain regions involving in motor control. Any damages to these regions will lead to motor function impairment. Both cerebellum and striatum receive input from and send signals to the cerebral cortex (Bostan et al., 2010). Recent data also suggest that these two regions are interconnected at the subcortical level and formed a complex integrated network with the cerebral cortex to coordinate motor, cognitive and affective functions (Bostan and Strick, 2018). Impairment in these interconnected regions will lead to abnormal motor and non-motor output, leading to diseases such as Parkinson’s disease, dystonia, and Huntington's disease (Bostan and Strick, 2018).

There are many forms of neuronal death which include death induced by various stimuli (e.g., excitotoxicity) or via different mechanisms that execute cell death (e.g., apoptosis, necrosis and autophagy) (Fricker et al., 2018). For example, amino acid glutamate is an excitatory neurotransmitter that potentially damages neurons via activation of glutamate-gated channels. Excitotoxicity is a pathological condition induced by excessive glutamate release from presynaptic nerve terminal or neighboring glial cells into extracellular spaces. Overactivation of glutamate receptors (ionotropic or metabotropic) by the high glutamate level leads to excessive calcium influx and disturbance of intraneuronal calcium homeostasis, thus causing neurons more susceptible to additional stress and subsequent neuronal death (Dong et al., 2009; Wojda et al., 2008).

The rapid influx and overload of calcium ions will trigger series of neurotoxic cascades, including failure in mitochondrial functions, production of reactive oxygen species (ROS), caspase activation, oxidative stress and cellular toxicity, which lead to neuronal damage and loss(Dong et al., 2009; Tehse and Taghibiglou, 2018). Excitotoxic neuronal damage due to uncontrolled and long-term exposure to excitatory amino acids such as glutamate plays a vital role in various neurodegenerative diseases (Olloquequi et al., 2018). Indeed, glutamate excitotoxicity has been implicated in multiple acute and chronic neurodegenerative diseases such as seizures, ischemic stroke, Alzheimer's disease and Parkinson's disease (Fu et al., 2018; Wang et al., 2020).

The kainic acid (KA) is widely used to induce excitotoxicity and epileptogenesis in animal model (Mohd Sairazi et al., 2015; Zheng et al., 2011). KA originated from seaweed *Digenea simplex* was initially used as an anthelmintic drug to treat ascariasis (a roundworm infection). It was later found that it can produce prolonged excitatory responses (Ben-Ari, 1985; Shinozaki and Konishi, 1970). Structurally, KA is an analogue of the excitatory neurotransmitter L-glutamate and acts as a potent agonist of kainate receptors, a type of ionotropic glutamate receptors which mediates fast excitatory neurotransmission (Zheng et al., 2011). It produces effects mimicking the behavioural and pathological changes in epilepsy (Vincent and Mulle, 2009).

Upon binding to kainate receptors, KA induces various cellular responses, including calcium influx, reactive oxygen species (ROS) production, endoplasmic reticulum stress and mitochondria dysfunction, leading to neuronal cell death in many brain regions (Zheng et al., 2011). Systemic administration of KA also induces seizures, behavioral and biochemical changes as well as neurodegeneration in various brain regions of rodent model (Mohd Sairazi et al., 2015; Reddy and Kuruba, 2013; Zheng et al., 2011). It was reported that after systemic administration of KA, oxidative injury were observed in various brain regions including hippocampus, cerebellum, amygdala/piriform cortex (Candelario-Jalil et al., 2001). KA-induced neurochemical changes in the amygdala/pyriform cortex, frontal cortex, striatum and substantia nigra were also observed up to 30 days after systemic KA injection (Sperk et al., 1986).

Excitatory amino acid transporter (EAAT) or glutamate transporter plays an essential role in glutamate reuptake and maintaining low extracellular glutamate levels.

Therefore, impairment in extracellular glutamate reuptake causes accumulation of glutamate in the synaptic cleft. Subsequently, overstimulation of glutamate receptors leads to high intracellular calcium ions levels, thus enhancing neuronal damage by excitotoxicity (Lewerenz and Maher, 2015; Magi et al., 2019). EAAT2, also known as glutamate transporter-1 (GLT-1) in rodents, is a major glutamate transporter responsible for approximately 80 – 90% of extracellular glutamate uptake activity in the brain (Kim et al., 2011). Studies showed that high expression of EAAT2 could significantly attenuate neuronal cell death, development of epilepsy and recurrent seizures (Kong et al., 2012; Lin et al., 2012). Therefore, restorations of EAAT2 expression level have a potential therapeutic role in neurodegenerative diseases by promoting reuptake of extracellular glutamate level and preventing neuronal cell damage (Pajarillo et al., 2019).

Many studies have been conducted to search for natural products useful in various diseases including neurodegenerative diseases. Honey is a natural food that has been used for centuries for nutritional and medical purposes. Apitherapy was developed by using honey and other bee products as an alternative treatment against various types of diseases including excitotoxicity-related neurodegenerative diseases (Bogdanov, 2016; El-Seedi et al., 2020). Tualang honey (TH) is a wild, multi-floral honey produced by Asian rock bees, *Apis dorsata*. It is rich in antioxidants such as quercetin and flavonoids that prevent oxidative stress and reduce neuronal damage and inhibit neuroinflammation in the brain (Qaid et al., 2020; Spagnuolo et al., 2018; Spencer and Crozier, 2012). TH was previously shown to protect against KA-induced oxidative stress and neuroinflammation in various brain regions of rats and reduced KA-induced

hyperactivity (Mohd Sairazi et al., 2018; Mohd Sairazi, K.N.S., Muzaimi, et al., 2017). However, the effects of TH on glutamate transporter remain unexplored. Therefore, this study aims to evaluate the effect of TH on the KA-induced behavioral and neuronal changes as well as the expression of EAAT2 (the predominant glutamate transporter) in the cerebellum and striatum of rats at different time points.

1.2 Hypothesis

This study hypothesized that TH exerts potential protective effects on KA-induced locomotor activity morphological changes, number of viable neurons and EAAT2 expression level on rat's cerebellum and striatum.

Null hypothesis (H_0): TH does not protect against KA-induced changes in locomotor activity, morphological changes, number of viable neurons and EAAT2 expression on rat's cerebellum and striatum.

Alternative hypothesis (H_A): TH protect against KA-induced changes in locomotor activity, morphological changes, number of viable neurons and EAAT2 expression on rat's cerebellum and striatum.

1.3 Objectives

1.3.1 General objectives

The general objective for this study were to evaluate the potential protective effect of TH on kainic acid (KA)-induced morphological changes and glutamate transporter (EAAT2) expression in the cerebellum and striatum of rats.

1.3.2 Specific objectives

The specific objectives for this study include:

I. To evaluate the effect of TH pre-treatment on locomotor behavioral activity in KA-induced excitotoxicity in rats after 24 hours and 5 days of KA administration.

II. To examine the effect of TH pre-treatment on histological changes in the cerebellum and striatum of KA-induced excitotoxicity in rats after 24 hours and 5 days of KA administration.

III. To examine the effects of TH pre-treatment on the expression of excitatory amino acid transporter 2 (EAAT2) in the cerebellum and striatum of KA-induced excitotoxicity in rats after 24 hours and 5 days of KA administration.

CHAPTER 2

LITERATURE REVIEW

2.1 Glutamatergic system

2.1.1 Glutamate

Glutamate is the most abundant excitatory amino acid in the CNS and mediates excitatory signals on the nerve cells (Nedergaard et al., 2002). It plays an essential role in higher-order brain functions including cognitive functions, learning and memory (Dauvermann et al., 2017; Gasbarri and Pompili, 2014). Upon depolarization, the glutamate stored in synaptic vesicles released via calcium-dependent exocytosis to the synaptic cleft. Glutamate exerts its neurotransmitter role by binding to its cell surface receptors which can be found throughout the brain (pre- and postsynaptic neurons and glial cells) (Watkins et al., 2008).

2.1.2 Glutamate receptors

Glutamate receptors can be classified into two major categories, the ionotropic and metabotropic receptor (Waxham, 2014) (Figure 2.1). Ionotropic receptors are multimeric, where protein subunits combine and forming a ligand-gated ion channel at the membrane (Kew and Kemp, 2005). Four main ionotropic glutamate receptors identified include N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate receptors (KAR) and delta receptors (Kumari et al., 2019; Meldrum, 2000). The binding of glutamate to these ligand-gated ion channels induces conformational changes and opening of ion channel that allows movement of ions across the membrane, produces excitatory postsynaptic potentials and mediate rapid (milliseconds) synaptic transmission (Sakakura et al., 2019; Traynelis et al., 2010). Glutamate also exerts its function indirectly through

metabotropic glutamate receptors. The binding of glutamate to these G-protein-coupled receptors mediate slower synaptic transmission than ionotropic receptors and can enhance or reduce neuronal excitability through modulation of intracellular second messengers. Metabotropic glutamate receptors (mainly Group II and III) also present at the axon terminals and function as autoreceptors to regulate glutamate release (Cartmell and Schoepp, 2000; Waxham, 2014)

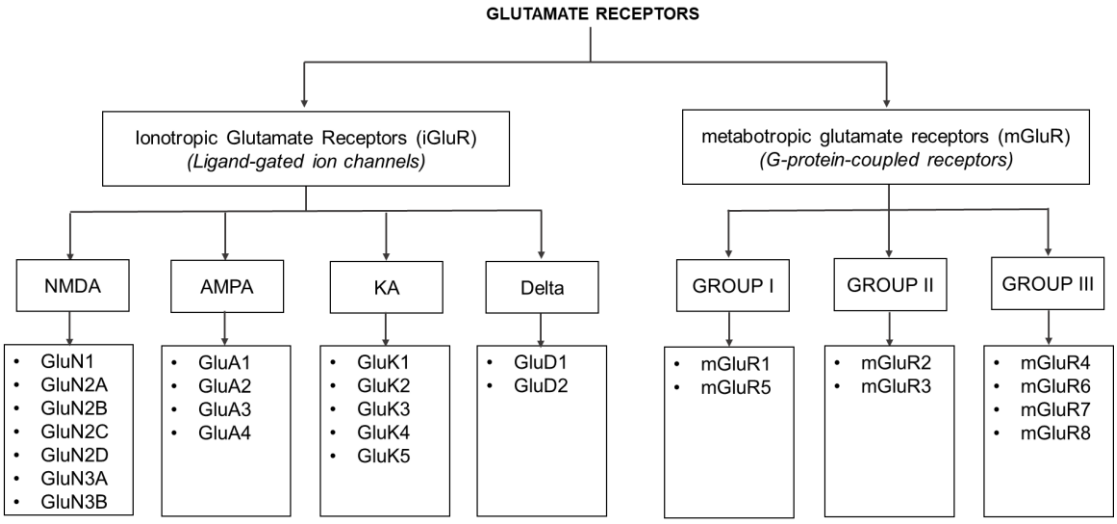

Figure 2.1 Glutamate receptor classifications[adapted from (Reiner and Levitz, 2018; Traynelis et al., 2010)] [Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; KA, kainate NMDA, N-methyl-D-aspartate]

KARs subunit are widely distributed in the brain. KARs subunit such as GluK1-3 can be found at high level in the striatum, granule cell layer of cerebellum, CA3 region of hippocampus and inner layers of cortex (Darstein et al., 2003; Herb et al., 1992; Nansen et al., 2000; Wüllner et al., 1997). KAR subunits GluK1 and GluK2 was also found in parallel fibers of cerebellum (Losada-Ruiz et al., 2019). KARs subunit such as GluK4 and GluK5 are distributed in specific region. GluK4 are found exclusively in hippocampus and GluK5 are found in striatum (Darstein et al., 2003; Gallyas et al., 2003). KARs in cerebellum involve in the modulation of biphasic effect on glutamate

release at low kainate concentration and depressed glutamate release at high kainate concentration at parallel fiber-purkinje cells synapse (Falcón-Moya et al., 2018).

Studies has demonstrated that KA direct activation on KAR plays important role in the imbalance of excitatory and inhibitory transmission which is associated with epilepsy (Losada-Ruiz et al., 2019). KARs containing GluK2 subunit are the common target for kainate to exerts convulsant effect inducing seizures and status epilepticus (Mulle et al., 1998). It was reported that non-NMDA receptors, AMPAR and KAR mediated glutamate excitotoxic effects on cultured striatal neurons, thus, might play greater role in excitotoxic death in disease and experimental animal models (Chen et al., 1995).

2.1.3 Glutamate transporters

The glutamate transporters, also known as excitatory amino acid transporters (EAATs), are responsible for extracellular glutamate re-uptake into neurons and non-neuronal cells (mainly glial cells) (O'Shea, 2002). The glutamatergic synapse under normal physiological conditions is illustrated in Figure 2.2.

Table 2.1 summarized the subtypes of EAATs and their predominant expression pattern in the CNS. These transporters control the excitatory synaptic transmission located near the synapse. Under normal conditions, the EAATs are sufficient to diminish the amount of glutamate released that is available for glutamate receptors activations (Diamond and Jahr, 1997; Huang and Zuo, 2005). The glutamatergic synapse under normal physiological conditions is illustrated in Figure 2.2.

Table 2.1 Excitatory amino acid transporters (EAATs) subtypes.

Subtypes	Synonym	Major CNS distribution	Major cell types
EAAT1	GLAST	Cerebellum, cortex, spinal cord	Astrocytes
EAAT2	GLT-1	Whole brain, spinal cord	Astrocytes
EAAT3	EAAC1	Hippocampus, cerebellum, striatum	Neurons
EAAT4	EAAT4	Cerebellum	Neurons (Purkinje cells)
EAAT5	EAAT5	Retina	Neurons (photoreceptors and bipolar cells)

References: (Seal and Amara, 1999; Zaitsev et al., 2020). [EAAC, excitatory amino acid carrier; EAAT, excitatory amino acid transporter; GLAST, glutamate aspartate transporter; GLT, glutamate transporter]

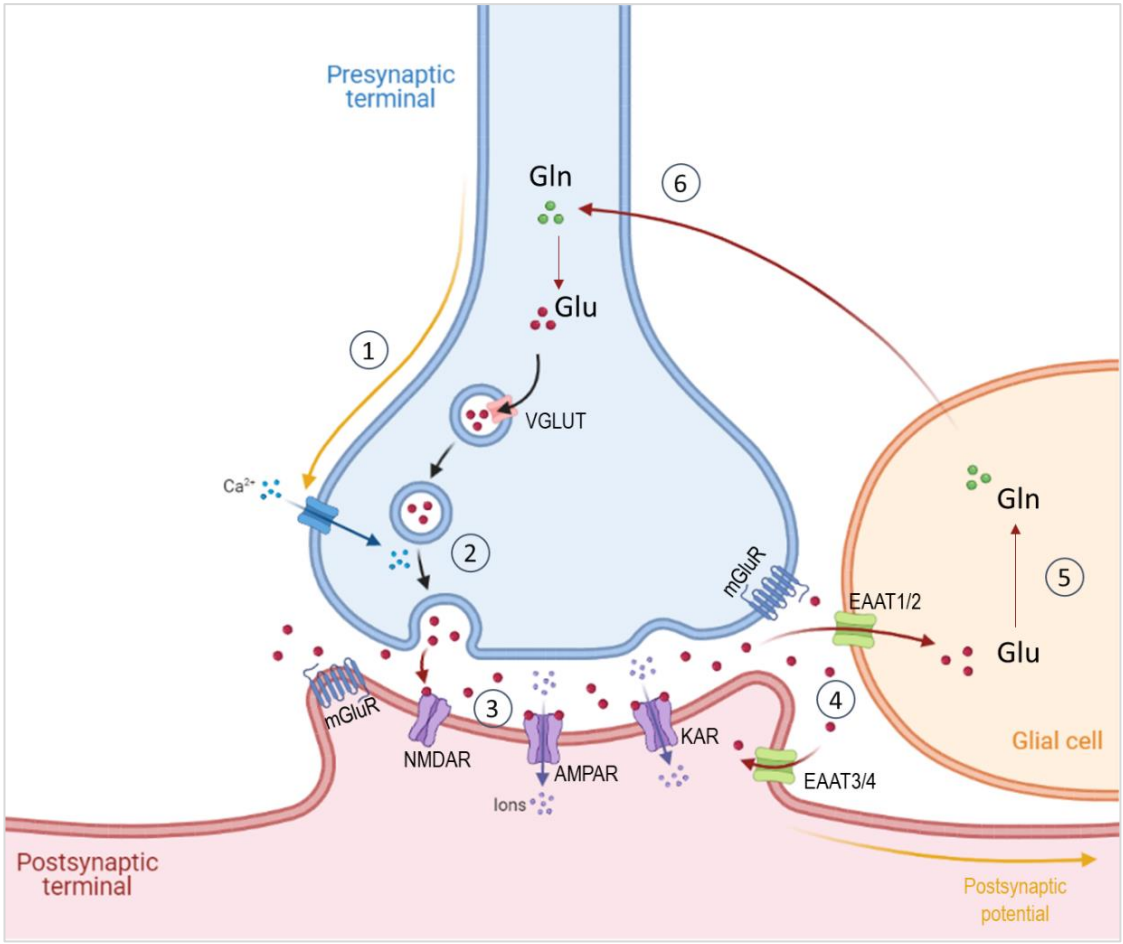

Figure 2.2 Glutamatergic neurotransmissions. [adapted from (Barker-Haliski and White, 2015; Bauer and Robinson, 2012; BioRender.com, 2021a; Sirohi and Kuhn, 2017)].

(1-2) Action potentials arrive at the axon terminal and trigger the opening of voltage-gated Ca^{2+} channel and cause vesicles to fuse with the presynaptic membrane and release glutamate into the synaptic cleft. **(3)** Binding of glutamate to ionotropic glutamate receptors (AMPAR, KAR, NMDAR) or metabotropic glutamate receptors (mGluR) to generate postsynaptic potential. **(4)** Unbound glutamate is cleared from the extracellular space by glial (EAAT1 or EAAT2) or neuronal (EAAT3 or EAAT4) glutamate transporters. **(5-6)** The glutamate was converted to glutamine by glutamine synthetase. This glutamine was then transported to the neurons and reconverted back into glutamate by glutaminase. Then, the glutamate is packaged in presynaptic vesicles by vesicular glutamate transporters and prepared to be released into the extracellular space.

[AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; KAR, kainate receptors; NMDAR, N-methyl-D-aspartate receptor; mGluR, metabotropic glutamate receptor; EAAT, excitatory amino acid transporter]

2.2 Glutamate excitotoxicity

The glutamate concentrations at the synaptic cleft are tightly regulated through multiple mechanisms for normal synaptic transmission and prevent continuos receptor stimulation. The extracellular glutamate concentrations are kept low by controlling glutamate release from the presynaptic neurons and cleared from the synaptic cleft via glutamate transporters (Altevogt et al., 2011). Glial cells (mainly astrocytes) also contribute to maintaining the glutamate concentrations via modulation of the glutamate-glutamine cycle. The astrocytic glutamate transporters take up excess glutamate in the synaptic cleft and convert it to glutamine by the enzyme glutamine synthetase (Bantle et al., 2021). Disturbances in the glutamatergic neuronal system can contribute to various pathological conditions.

Glutamate excitotoxicity is a pathological condition when there is an abnormal elevation in extracellular glutamate levels and overactivation of postsynaptic glutamate receptors (Sheldon and Robinson, 2007). It can be due to excessive glutamate release from the presynaptic neurons or impaired glutamate uptake by the glial cells, which in turn causes excessive accumulation of glutamate in the synaptic cleft (Mattson, 2019). Overstimulation of glutamate receptors such as NMDA, AMPA and kainate receptors leads to a high influx of Ca^{2+} in postsynaptic neurons and triggers activation of several events that lead to neuronal death (Simonian et al., 1996). Some of these include activation of endonucleases and proteases, mitochondrial dysfunction, oxidative stress and inflammation that eventually lead to neuronal death (Vishnoi et al., 2016).

In addition, excessive glutamate also promotes glutamate spillover from the synapses, leading to non-specific glutamate binding at extrasynaptic receptors and inducing abnormal excitatory transmission which eventually causes neuronal death (Lewerenz and Maher, 2015). Therefore, it is crucial to control extracellular concentrations of glutamate to maintain the excitatory neurotransmitter level and prevent excitotoxic-induced neuronal death (Sheldon and Robinson, 2007; Vandenberg and Ryan, 2013).

Glutamate excitotoxicity has been linked to various NDD such as trauma, epilepsy, Parkinson's disease, Huntington disease and Alzheimer's disease (Zheng et al., 2011). For example, the imbalance of excitatory action by the glutamate and inhibitory action by gamma-aminobutyric acid (GABA) lead to hyperexcitability in neurons and epilepsy (Hui Yin et al., 2013). Glutamate plays a role in inducing epileptogenesis due to its ability to overstimulate glutamate receptors, causes long-term seizure activity and neuronal damage in the brain (Barker-Haliski and White, 2015).

2.3 Kainic acid-induced excitotoxicity animal experimental model

Kainic acid (KA) was named after *kaininso,* the Japanese name of the red seaweed *Digenea simplex* from which it is isolated. This marine red alga can be found in tropical and subtropical waters (Murakami et al., 1953). KA was initially used as an anthelminthic agent. Since the discovery of its ability to activate a type of glutamate receptor, KA has been commonly used to induce excitotoxicity in various neurodegenerative models to understand the underlying mechanisms involved in neurodegeneration. Studies of *in vivo* KA-induced excitotoxicity have shown that KA induces neuronal cell death via apoptosis, necrosis, autophagy and programmed cell death (PCD) (Tokuhara et al., 2007; Wang et al., 2005).

KA is an analogue of glutamate that acts as a potent agonist of AMPA and kainate receptors, two subtypes of ionotropic glutamate receptor family which plays an essential role in regulating physiological activities in various brain regions (Vincent and Mulle, 2009). It is a neuronal excitant and causes neurotoxicity 30-fold more potent than glutamate (Zhang and Zhu, 2011). Widespread neuropathological changes in various brain regions were evidenced following systemic KA administration, including the striatum, hippocampus, cerebral cortex, piriform cortex, and amygdala (Candelario-Jalil et al., 2001; Lintunen et al., 2005; Mohd Sairazi et al., 2017; Sperk et al., 1986).

Upon binding to the glutamate receptor, KA causes the opening of the ligand-gated ion channels, membrane depolarization and calcium entry through voltage-gated ion channels (Warren et al., 2016). Overactivation of these receptor lead to excitotoxicity where high intracellular calcium levels trigger activation of calcium-dependent

enzymes and free radical production, followed by mitochondrial dysfunction and ATP depletion which eventually led to neuronal death and functional loss (Dong et al., 2009; Prentice et al., 2015; Tian et al., 2019; Zhang and Zhu, 2011). The KA-induced neuronal damage was illustrated in Figure 2.3.

Excitotoxicity has been implicated in various neurodegenerative diseases including Parkinson's disease, Alzheimer's diseases, Huntington's disease, cerebral ischaemia and epilepsy (Ezza and Khadrawyb, 2014; Lewerenz and Maher, 2015). Kainic acid (KA) is commonly used to induce excitotoxicity and epilepsy in animal experimental models. KA causes neuronal death in rat brain and has been used as a model to understand the pathogenesis of excitotoxicity in neurodegenerative diseases (Zhang and Zhu, 2011; Zheng et al., 2011). KA was also commonly used to study epileptogenesis and neuropathological changes associated with seizure onset and neurodegeneration in the brain regions. KA-induced temporal lobe epilepsy using animal models was shown to produce neuropathological, behavioural and electroencephalographic features similar to those seen in human patients (Ben-Ari, 1985).

Behavioral changes were also observed upon KA administration, including limbic seizures with characteristics of wet-dog shake, facial and forelimb clonus and rearing and falling in KA animal model (Lévesque and Avoli, 2013). Studies have also reported that upon KA injection causes hyperactivity can lead to impairment in spatial learning and object exploration tasks also less immobility and higher locomotor observed in KA-induced rats. (Riljak et al., 2015; Zheng et al., 2011).In addition, the

hyperactivation of glutamate receptors due to KA can lead to loss of motor neurons and progressive impairment in motor-selective behaviour (Sun et al., 2006).

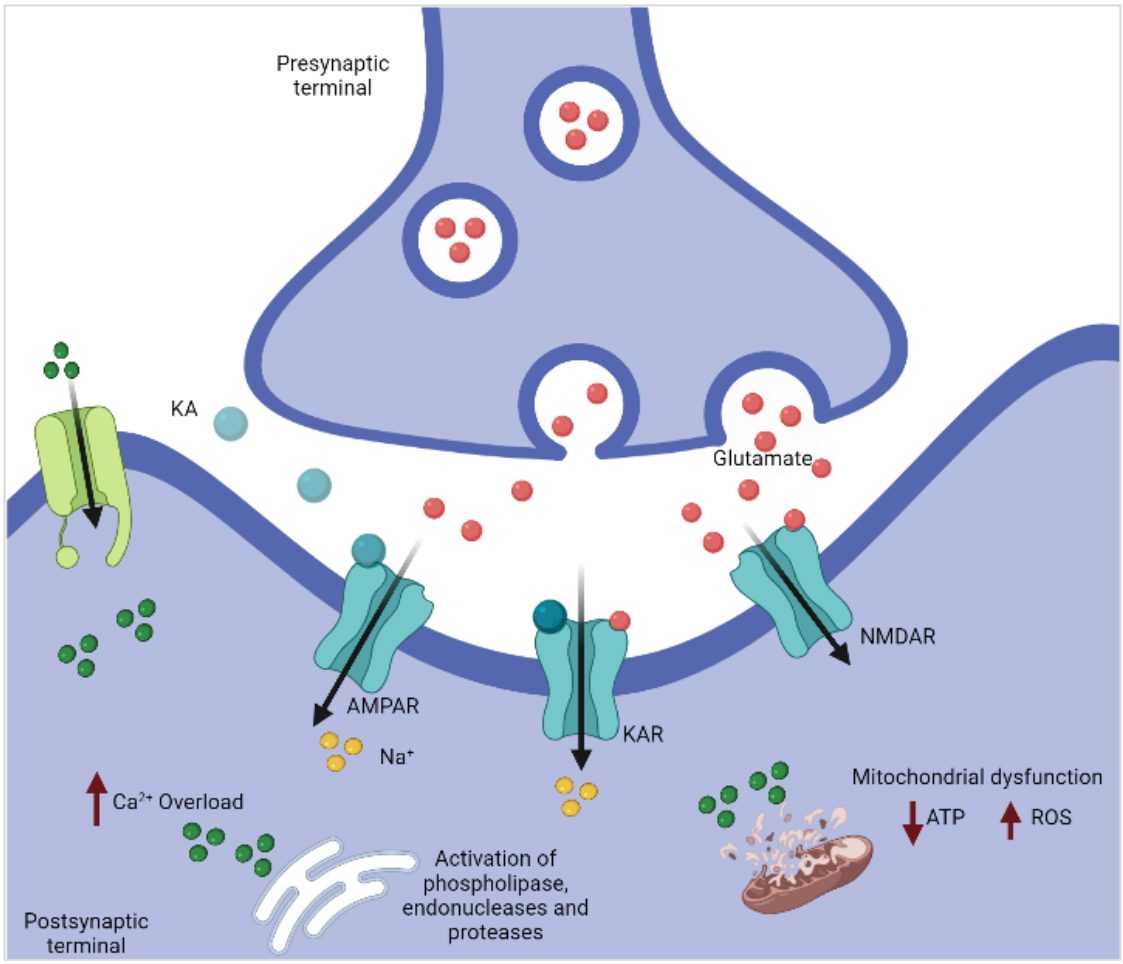

Figure 2.3 Diagram of mechanisms involves in KA-induced neuronal damage.

KA binds to AMPA or kainite receptors, causes the opening of the ligand-gated ion channels, membrane depolarization and calcium entry through voltage-gated ion channels. Thus, overactivation of glutamate receptors will lead to high intracellular calcium levels that trigger activation of calcium-dependent enzymes such as phospholipase, endonucleases and proteases. Moreover, high calcium influx will lead to mitochondrial damage, free radical production and ATP depletion, leading to neuronal death and functional loss. [AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ATP, adenosine triphosphate; ER, endoplasmic reticulum; KA, kainic acid; KAR, kainate receptor; NMDAR; N-methyl-D-aspartate receptor; ROS, reactive oxygen species] [adapted from (BioRender.com, 2021b; Rusina et al., 2021; Sirohi and Kuhn, 2017)].

2.4 Role of EAAT2 in neurodegenerative diseases

EAAT2 (or GLT-1) is expressed predominantly in astrocytes throughout the brain, and less than 10% is expressed neuronally (Sharma et al., 2019; Todd and Hardingham, 2020). EAAT2 is the major type of glutamate transporter responsible for approximately 80 – 90% of extracellular glutamate uptake activity in the brain, hence plays a central role in glutamate clearance and prevent excitotoxicity (Kim et al., 2011). Moreover, EAAT2 maintaining synaptic glutamate concentration levels also critical in regulating normal glutamatergic transmission activity (Pita-Almenar et al., 2012). Several studies have demonstrated that the upregulation of the EAAT2 expression level can produce protective effects against neurological disorders (Takahashi et al., 2015). Therefore, studies in EAAT2 provide more insights as a potential therapeutic target for several diseases.

Any dysfunctions or reduced expression in EAAT2 may lead to impaired synaptic glutamate clearance and subsequent accumulation of extracellular glutamate will cause a wide range of NDD (Malik and Willnow, 2019). For example, EAAT2/GLT-1 knockout mice shown to be more susceptible to acute brain injury and spontaneous lethal seizures associated with slower clearance of glutamate than wild-type mice, indicating the importance of glial glutamate transporters in the maintenance of low synaptic glutamate to prevent hyperexcitability, brain injury and epilepsy. The study showed that half of the EAAT2 knockout mice died prematurely (before six weeks), and the mortality rate was about 80% by the age of 13 weeks, whereas all wild-type mice survived (Tanaka et al., 1997). Meanwhile, the brain of EAAT1 knockout mice appears to develop normally but are more susceptible to brain injury (Watase et al., 1998). Double EAAT1/EAAT2 knockout mice showed early embryonic death and

exhibited widespread brain structure abnormalities (Matsugami et al., 2006). These findings support the critical roles of astrocytic glutamate transporters in the brain, especially the EAAT2 subtype.

In contrast, studies have also shown that overexpression of EAAT2 exerts neuroprotective roles by reducing extracellular glutamate concentration and neuronal cell damage in various types of NDD such as epilepsy, ischemia, and traumatic brain injury (Lin et al., 2012). For example, overexpression of EAAT2 in kainic acid-induced transgenic mice displayed less severe seizures as well as a reduction in cell death and increased survival, supporting the neuroprotection conferred by EAAT2 (Martinowich et al., 2001; Scholz and Sutherland, 2003). Moreover, inducing EAAT2 translation might be a potential therapeutic strategy where it exerts neuroprotective effects by preventing excitotoxicity, delayed loss of motor functions, neuronal cell damage and recurrent spontaneous seizures were observed in the pilocarpine epileptic model (Kong et al., 2014). Furthermore, upregulation of EAAT2 expression also plays an essential role in the ischaemia-induced rat model by significantly reducing excessive glutamate concentration, neuronal cell damage and improving behavioral activity (Harvey et al., 2011).

2.5 Cerebellum and striatum

The cerebellum and striatum are the major subcortical structures that form interconnected networks with the cerebral cortex, and the neuronal activity in these regions involve in movement performances (Strick et al., 2009). The cerebellum is connected to the brainstem and located at the back of the brain, on top of the pons (Rapoport et al., 2000). The cerebellum can be divided into three anatomical lobes: the anterior, posterior and flocculonodular lobes. Functionally, it can be divided into three zones: the vestibulocerebellum, spinocerebellum and cerebrocerebellum) (Roostaei et al., 2014). The cerebellum is involved in motor performances and plays a crucial role in coordinating voluntary movement, cognitive functions, motor learning and balance maintenance (Rapoport et al., 2000).

The striatum is one of the main inputs of basal ganglia that receives afferent fibers from several brain regions to regulate voluntary movement (Bonsi et al., 2011; Watson et al., 2010). The three primary striatal afferent fibres are the corticostriatal, thalamostriatal and nigrostriatal pathways that influence neurons in the thalamus, which then project back to the cerebral cortex. Striatum identifies and organizes specific activity combinations of the cortical circuits to limit overlapping of cortical projections from diverse sources (Wilson, 2009). The location of the cerebellum and striatum are illustrated in Figure 2.4.

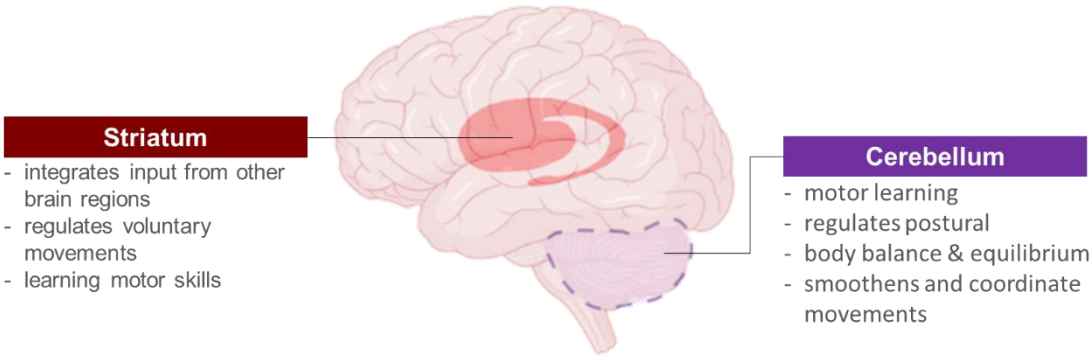

Figure 2.4 Location of the cerebellum and striatum [adapted from (Balleine et al., 2007; Ghandili and Munakomi, 2020; Yanagihara, 2014)].

Various neurological diseases such as Alzheimer's disease, Parkinson's disease, stroke and epilepsy share the same clinical symptoms such as gradual loss or deterioration in motor performances (Dugger and Dickson, 2017; Kovacs, 2018). The cerebellum and striatum play important roles in motor performances including motor learning, motor control and motor skills acquisition and execution via cortical-subcortical circuits (Centonze et al., 2008). Damages to these two regions can cause clinical significance in dysfunctional motor and attention performances (Volpe, 2012).

Various neurotransmitters such as serotonin, dopamine, glutamate and gamma-aminobutyric acid (GABA) interact in these regions to regulate excitatory or inhibitory neurotransmission of motor neurons in these circuitries (Abg Abd Wahab et al., 2019). In the cerebellum, excitatory inputs are projected from granular neurons, then the Purkinje cells project inhibitory signals to the cerebral cortex (Roostaei et al., 2014). Glutamatergic and dopaminergic inputs received by striatum will be transmitted to output structures of basal ganglia via direct and indirect pathways (David, 2009). Striatum is usually associated with Parkinson's disease and epilepsy which involves impairments in inhibitory and excitatory neurotransmission (Miyamoto et al., 2019;

Zhai et al., 2018). Studies has shown that Parkinson's disease is associated with higher risk of having epileptic seizures (Gruntz et al., 2018).

Neuroimaging studies showed significant blood flow changes in the cerebellum and striatum during learning and automatization of motor skills (Doyon, 1997). A study showed that adaptive changes in striatal synaptic transmission compensated cerebellar damage and facilitate recovery from motor deficits, supporting the interconnection between these two essential regions in motor function (Centonze et al., 2008). Therefore, changes at one node of either region will affect the whole network, thus influencing the action on the other related node (Bostan and Strick, 2018). Studies has reported that these regions were affected after kainic acid administration (Candelario-Jalil et al., 2001; Lintunen et al., 2005; Sperk et al., 1986). KA-experimental model also showed that it induce seizures, injuries in various brain regions and behavioural changes (Milatovic et al., 2002; Mohd Sairazi et al., 2017; Swamy et al., 2011).

2.6 Honey

Apitherapy is a type of complementary medicine that uses honey or other bee products as an adjunctive treatment against various diseases (Bogdanov, 2016). Bee products such as propolis, bee pollen, honey royal jelly and bee venom consist of bioactive ingredients and nutrients with many health benefits. Bee products have been used for decades in different countries such as Egypt, China and Japan as dietary supplements and medicines (Ali and Kunugi, 2020).

Honey is a natural sweetener produced by honeybees from sugar-rich exudates from plants. Floral honey, also known as blossom or nectar honey, is made from the nectar of the blossomed flower from a single or multiple flower source. It takes approximately two million flowers to produce one pound of honey. Meanwhile, honeydew honey is made from sap produced by certain plants or the secretion from insects that suck the plant's sap. (Pita-Calvo and Vázquez, 2017). Both collected nectar and honeydew were then transformed and stored in the honeycomb to ripen and mature (Saba et al., 2013). Honey has been used for centuries for nutritional and medical purposes by our ancestors.

2.6.1 Composition and health benefits of honey

The composition of honey varies depending on the botanical and geographical origin. The honey colour ranges from nearly colourless to dark amber or black, depending on their floral sources and compositions (Olaitan et al., 2007). The major constituent of honey is sugars (up to 95 to 99% of honey's dry substance) which include fructose (38%), glucose (31%) and other sugars as energy providers (Alnaqdy et al., 2005; El Sohaimy et al., 2015). Other components found in honey include water, phenolic compounds, organic acids, vitamins and minerals, enzymes such as catalase and glucose oxidase, free amino acids, Maillard reaction products and volatile compounds (Eteraf Oskouei and Najafi, 2013).

Honey is known to exert various beneficial medicinal properties, including wound healing potential, antibacterial, antioxidants and anti-inflammatory properties (Vallianou, 2014). For instance, honey demonstrated anti-inflammatory effects comparable to prednisolone in an inflammatory model of colitis by preventing the free radicals production released by the inflamed tissues (Bilsel et al., 2002). Honey is also shown to have a soothing action on the mucus membrane of oral mucositis due to its ability to inhibit bacterial and fungal growth and enhanced wound healing (Liza and Ovington, 1999; Wang et al., 2012). Besides, honey showed neuroprotective effects in reducing neuroinflammation in the ischemia-induced rat model (Zarraga-Galindo et al., 2011). A Nigerian honey from Umudike region also showed anticonvulsant effects against picrotoxin-induced seizure model by delaying onset of seizures and reduced number of seizures in mice (Akanmu et al., 2011).